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A Nucleotide That Enhances the Charging of RNA Minihelix Sequence Variants with Alanine†

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ABSTRACT: We showed earlier that a single G3·U70 base pair within the amino acid acceptor helix is a major determinant of the identity of $tRNA^{Ala}$. In addition, we demonstrated that an RNA hairpin minihelix that recreates the 12 base pair acceptor–TψC stem of $tRNA^{Ala}$ is also aminoacylated in a G3·U70-dependent manner. Determinants for efficient aminoacylation at pH 7.5 have been further investigated with minihelix substrates that have sequence variations at 3·70 and other locations. Although a U,U mismatch and other 3·70 nucleotide alternatives to G·U were recently proposed by others as also important for alanine acceptance, neither that mismatch nor any of four other 3·70 nucleotide combinations confer aminoacylation in vitro with alanine, even with substrate levels of enzyme. In contrast, permutations of the so-called discriminator nucleotide N73 (at position 73) strongly modulate, but do not block, aminoacylation of those substrates that encode G3·U70. In particular, the efficiency of G3·U70-dependent aminoacylation with alanine is strongly enhanced by having the wild-type A73. The effect of N73 alone can explain most of the difference in aminoacylation efficiency of a G3·U70-containing tRNA and a minihelix substrate whose sequences vary significantly from their $tRNA^{Ala}$ counterparts. Comparison with earlier work suggests that the substantial modulating effect of N73 is partly or completely obscured when N73 tRNA variants are expressed as amber suppressors in vivo.

For cells to preserve the fidelity of protein synthesis, tRNAs must be aminoacylated exclusively with their cognate amino acids. Recently, the sequence elements involved in establishing the identity of a tRNA have been investigated for several different tRNAs (Schimmel, 1989; Normanly & Abelson, 1989). From these studies, the acceptor stem and the anticodon have emerged as two regions where identity elements are concentrated (Hou & Schimmel, 1988; Schulman & Pelka, 1988, 1989; Sampson et al., 1989; Francklyn & Schimmel, 1989; Rould et al., 1989). In $tRNA^{Ala}$, the G3·U70 base pair in the amino acid acceptor helix acts as a major determinant of alanine identity (Hou & Schimmel, 1988; Francklyn & Schimmel, 1989; Park et al., 1989; Hou & Schimmel, 1989a,b). Because G3·U70 is unique to $tRNA^{Ala}$ (Sprinzl et al., 1989), it can in principle be used to distinguish that tRNA from all others. When introduced into $tRNA^{Cys}$, $tRNA^{Phe}$, and $tRNA^{Tyr}$ amber suppressors, the G3·U70 base pair confers on each of the host tRNAs the ability to insert alanine at a UAG codon (Hou & Schimmel, 1988, 1989b; McClain & Foss, 1988). In addition, RNA footprinting studies demonstrated that alanine tRNA synthetase protects nucleotides along the 3′ side of the acceptor stem and the unpaired CCA terminus of $tRNA^{Ala}$, but does not shield the anticodon at all (Park & Schimmel, 1988).

More recently, we demonstrated that small synthetic RNA helices whose sequences are based on a limited portion of the sequence of $tRNA^{Ala}$ are excellent substrates for alanine tRNA synthetase. A minihelix based on the acceptor–TψC stem of $tRNA^{Ala}$ is efficiently aminoacylated at a rate comparable to that of $tRNA^{Ala}$ (Francklyn & Schimmel, 1989). Substitution of G·C for the G3·U70 base pair abolishes aminoacylation with alanine. Also, transplantation of the G3·U70 base pair into a minihelix based on the acceptor–TψC sequence of $tRNA^{Tyr}$ confers alanine acceptance in vitro. Significantly, the G3·U70 $minihelix^{Tyr}$ has almost the same kinetic parameters for aminoacylation as a full-length $tRNA^{Tyr}$ into which the G3·U70 base pair has been transplanted (Francklyn & Schimmel, 1989; Hou & Schimmel, 1989b). This suggested that, in full-length $tRNA^{Tyr}$, the 49 nucleotides that are outside the acceptor–TψC stem do not perturb the interaction of the enzyme with the minihelix segment (acceptor–TψC stem) of the tRNA.

Because the minihelix substrates respond to substitutions at the 3·70 position in a manner indistinguishable from that of the full-length tRNA, we have used them to investigate further the basis of molecular recognition between $tRNA^{Ala}$ and its cognate synthetase. In particular, prior work has left unresolved two important questions concerning the role of the G3·U70 base pair in establishing alanine identity. First, McClain et al. (1988) observed weak in vivo suppression of an amber codon in a β-galactosidase mRNA by variants of $tRNA^{Ala}$ that encode alternative bases at the 3·70 position, including the U3·G70 transversion and the U3,U70 mismatch. This raised the possibility that a helical irregularity at the 3·70 position can, to a low extent, confer alanine acceptance. However, studies with the U3·G70 variant of $tRNA^{Ala}$, and with the A3·U70 and G3·C70 variants, failed to demonstrate any in vitro aminoacylation with alanine, even after prolonged incubation of each variant with substrate levels of enzyme

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(Park et al., 1989). These observations have prompted further studies by us to determine whether a helical irregularity at the 3·70 position is a major contributor to the identity of $tRNA^{Ala}$. For this purpose, we have made minihelix substrates to explore the capacity for aminoacylation of variants that have mismatches such as U3,U70 and G3,G70.

The second question concerns the extent to which nucleotides other than G3·U70 modulate the interaction of alanine tRNA synthetase with its substrate. Six different tRNA sequence contexts have been investigated in vitro as substrates for the enzyme (Hou & Schimmel, 1988, 1989a,b; Park et al., 1989). In each case, aminoacylation is conferred by the G3·U70 base pair, but the relative efficiencies are different. The most dramatic example is the G3·U70 $tRNA^{Cys}$ amber suppressor. While this tRNA is an unusually efficient alanine-inserting amber suppressor in vivo, it has by far the lowest aminoacylation efficiency in vitro of the six substrates that have been tested (Hou & Schimmel, 1988). To investigate the cause of the low aminoacylation efficiency, we first showed that a minihelix comprising the acceptor–TψC stem of G3·U70 $tRNA^{Cys}$ is a substrate for alanine tRNA synthetase and that its aminoacylation efficiency was sharply reduced compared to other G3·U70 minihelix substrates. This suggested that the reduced in vitro aminoacylation efficiency of G3·U70 $tRNA^{Cys}$ was due to the effect of one or more nucleotides in the acceptor–TψC stem that, in turn, could be identified by investigation of the minihelix substrates.

Materials and Methods

Oligodeoxynucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer in the MIT Center for Protein Structure and Engineering and were then purified by electrophoresis and elution over a Waters Sep-Pak column. The purified DNA was stored in 10 mM Tris-HCl (pH 8.0) at −20 °C. Nucleoside triphosphates were purchased from Pharmacia/P-L Biochemicals, dissolved in water (adjusted to neutral pH with NaOH), and then stored at −20 °C. T7 RNA polymerase was either purchased from U.S. Biochemicals or purified from *Escherichia coli* strain BL-21/pAR 1219 (Davanloo et al., 1984). *E. coli* $tRNA^{Ala}$ (UGC isoacceptor) was purchased from Subriden RNA. [^{3}H]Alanine (84 Ci/mmol) was from NEN Du Pont. *E. coli* alanine tRNA synthetase was purified (Regan, 1986) to ≥90% homogeneity, and the concentration was determined by active-site titration (Fersht et al., 1975).

RNA synthesis was carried out as described (Milligan et al., 1987; Sampson & Uhlenbeck, 1988). The primer and template DNAs were annealed at a concentration of 10 μM (for both fragments) by heating together at 80 °C for 4 min in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) buffer (Sambrook et al., 1989) and then slowly cooled to room temperature. The transcription reactions were carried out at 37 °C in a reaction mixture containing 0.5 μM DNA, 40 mM Tris-HCl (pH 8.1), 1 mM spermidine, 5 mM dithiothreitol, 50 μg/mL bovine serum albumin, 0.01% (v/v) Triton X-100, 80 mg/mL poly(ethylene glycol) (M_r 8000), 4 mM of each nucleoside triphosphate, 20 mM $MgCl_2$, and 2.5 units/μL T7 RNA polymerase. Following the 4-h synthesis, the reactions were terminated by phenol extraction and ethanol precipitation and then fractionated on denaturing 20% polyacrylamide gels.

The aminoacylation reactions (Schreier & Schimmel, 1972) were carried out at 37 °C in a reaction mixture containing 50 mM phosphate buffer (pH 7.5), 20 mM KCl, 0.1 mg/mL bovine serine albumin, 20 mM β-mercaptoethanol, 22.4 μM [^{3}H]alanine, 4 mM ATP, and concentrations of minihelix and alanine tRNA synthetase that are indicated in the figure legends. For determinations of kinetic parameters, RNA concentrations were varied over a 64-fold range. RNA concentrations were calculated on the basis of the maximum concentration of molecules which could be aminoacylated when time and synthetase concentration were not limiting. For nonacylatable substrates, concentrations of one absorbency unit at 260 nm were set equal to 35 μg/mL [cf. Francklyn and Schimmel (1989)].

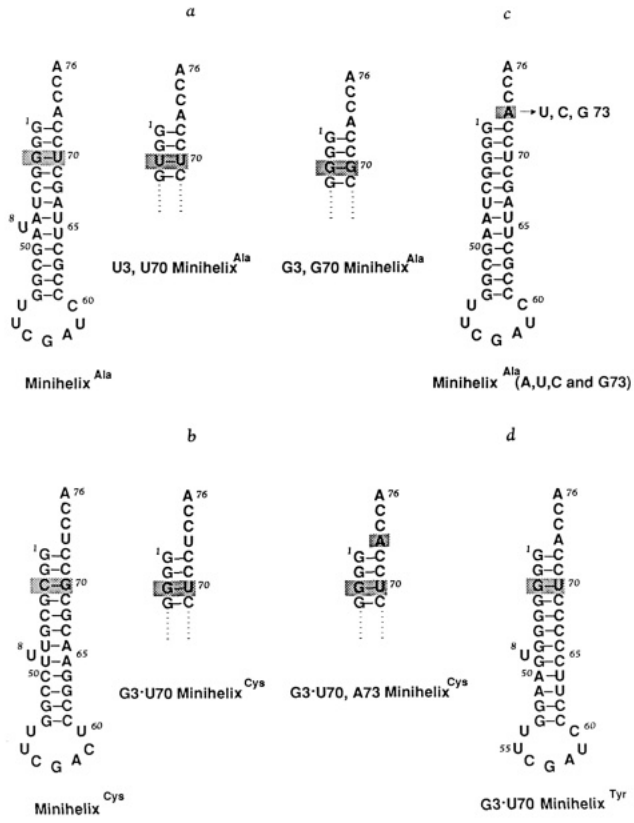

FIGURE 1: Synthetic RNA minihelices based on the acceptor–TψC helix of tRNA. Numbering is based on that of the corresponding intact tRNA. The shaded portions refer to the positions where substitutions have been made. (a) Wild-type, G3,G70, and U3,U70 variants of $minihelix^{Ala}$. (b) Wild-type, G3·U70, G3·U70,A73, and A73 variants of $minihelix^{Cys}$. (c) $Minihelix^{Ala}$ with U8 deleted, along with U73, G73, and C73 variants. (d) G3·U70 $minihelix^{Tyr}$. The wild-type $minihelix^{Tyr}$ has a U3·A70 base pair.

Results

Design of Minihelix Substrates for Alanine tRNA Synthetase. Three different sets of minihelix substrates were used in this work. Each minihelix in Figure 1 is based on the acceptor–TψC sequence of a tRNA and contains 12 base pairs, a 7-nucleotide loop, and the 4 unpaired nucleotides at the 3′ end. The minihelices in Figure 1a were derived from the sequence of $tRNA^{Ala}$ and test the effect of G,G or U,U mismatches at the 3·70 position. The sequence of $tRNA^{Cys}$ was used to generate the minihelices in Figure 1b. The variants in this set were designed to examine the relative contributions of G3·U70 and A73 to alanine acceptance. In the substrates shown in Figure 1c, substitutions in $minihelix^{Ala}$ were introduced at position 73 [sometimes called the discriminator base (Crothers et al., 1972)]. By use of the in vitro transcription protocol described under Materials and Methods, samples of each of these minihelices were prepared and then used as substrates in aminoacylation assays.

It should be noted that the minihelix substrates were made with and (later) without the unpaired uridine at position 8. The internucleotide phosphate on the 3′ side of U8 (in

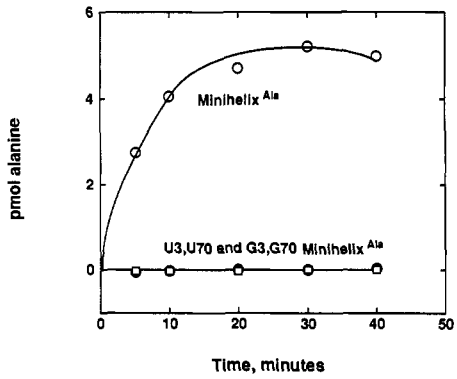

FIGURE 2: Aminoacylation with alanine of minihelix^{Ala} and variants. Assay conditions were as described under Materials and Methods. For U3,U70 and G3,G70 variants of minihelix^{Ala}, the RNA concentrations were 2 μM, and alanine tRNA synthetase concentrations were 0.75 μM. For minihelix^{Ala}, the RNA concentration was 0.3 μM, and the concentration of alanine tRNA synthetase was 75 nM. The incorporation of alanine per 18-μL reaction aliquot is given on the ordinate axis.

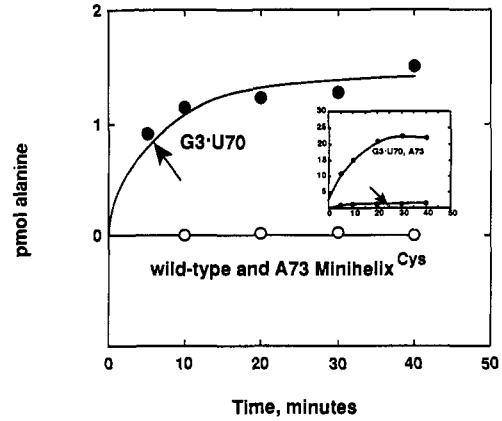

FIGURE 3: Aminoacylation of minihelix^{Cys} and its variants. Assays were carried out as described under Materials and Methods. For wild-type and A73 minihelix^{Cys}, the RNA and alanine tRNA synthetase concentrations were 1 and 1.1 μM, respectively. For G3·U70 and G3·U70,A73 variants, the RNA concentrations were 2 and 1.25 μM, and alanine tRNA synthetase concentrations were 750 and 75 nM, respectively. The inset shows data for G3·U70,A73 minihelix^{Cys}, and for comparative purposes replots the data for G3·U70 minihelix^{Cys} (marked with an arrow). The incorporation of alanine per 18-μL reaction aliquot is given on the ordinate axis.

tRNA^{Ala}) is protected by bound alanine tRNA synthetase from nuclease digestion (Park & Schimmel, 1988) and was easy to incorporate in the first minihelices [cf. Francklyn and Schimmel (1989)]. However, we found that the K_m and k_{cat} parameters for alanine minihelices were unaffected by the presence of the bulged U8 (shown below). For this reason, we discontinued its incorporation into subsequent minihelix substrates.

Minihelices with U3,U70 and G3,G70 Mismatches Are Not Aminoacylated in Vitro by Alanine tRNA Synthetase. Some of the position 3·70 sequence variants investigated by McClain et al. (1988) are weak amber suppressors that insert alanine in addition to other amino acids. Among these, the U,U mismatch preferentially inserted alanine at an amber codon in the mRNA of dihydrofolate reductase. In the case of the G,G mismatch, the resulting dihydrofolate reductase protein was not analyzed.

After a 3-h incubation period with 2 μM of each substrate and with relatively high levels of alanine tRNA synthetase (0.75 μM), neither of the two minihelix variants incorporated [^{3}H]alanine above background levels (Figure 2). Under the same conditions, minihelix^{Ala} (which encodes G3·U70) was quantitatively aminoacylated. It is of interest to note that the U3,U70 variant fails to aminoacylate, even though, in principle, it retains the potential to contact the enzyme at U70 [as observed in the RNA footprint (Park & Schimmel, 1988)]. We conclude that a mismatch at position 3·70 per se is not sufficient for aminoacylation in vitro under standard conditions.

The Base at Position 73 Strongly Influences the Extent and Rate of Aminoacylation of Variants of G3·U70 Minihelix^{Cys}. The acceptor–TψC region of tRNA^{Cys} differs from that of tRNA^{Ala} at 5 of 12 base pairs and at position 73, which is a U instead of an A. Of the six tRNA sequence contexts investigated in vitro with alanine tRNA synthetase, G3·U70 tRNA^{Cys} is the only one that has a nucleotide other than A at position 73 and is the only one to be significantly reduced in aminoacylation efficiency (Hou & Schimmel, 1988; Park et al., 1989; Hou & Schimmel, 1989a,b). For example, G3·U70 minihelix^{Tyr} (Figure 1d), which is almost equivalent to G3·U70 tRNA^{Tyr} as a substrate (Francklyn & Schimmel 1989; Hou & Schimmel, 1989b), differs at 7 of 12 base pairs from minihelix^{Ala}, but retains A73. This suggested a role for the position 73 nucleotide in determining the efficiency of aminoacylation with alanine.

To investigate whether the effect of context on recognition of the G3·U70 base pair is restricted to the acceptor–TψC region, the appropriate minihelix^{Cys} variants were synthesized (Figure 1b). As shown in Figure 3, aminoacylation of minihelix^{Cys} with alanine was dependent on the introduction of G3·U70. However, the extent of aminoacylation of this substrate was low, such that aminoacylation was only observed with substrate amounts of synthetase. [Poor substrates are typically not completely aminoacylated, presumably because of competing editing reactions (Dietrich et al., 1976; Schreier & Schimmel, 1972).] Thus, the data cannot be used to determine accurate kinetic parameters. The poor aminoacylation observed with this minihelix substrate recapitulates the slow aminoacylation of the full-length G3·U70 tRNA^{Cys} (Hou & Schimmel, 1988). This suggests that the defect in aminoacylation lies within the acceptor–TψC stem. This finding is also consistent with the previous observation that sequences outside the acceptor–TψC region of G3·U70 tRNA^{Tyr} do not perturb the interaction of alanine tRNA synthetase with the acceptor helix (Francklyn & Schimmel, 1989; Hou & Schimmel, 1989b).

Figure 3 shows that introduction of A73 into G3·U70 minihelix^{Cys} dramatically increases the aminoacylation efficiency. The k_{cat} value is reduced only 3-fold relative to the values for tRNA^{Ala} and minihelix^{Ala} (Table I). Because a major part of discrimination is at the k_{cat} step, this result suggests that the difference in the number of nucleotides and in the sequence of G3·U70,A73 minihelix^{Cys} versus tRNA^{Ala} are not important for passage of the enzyme–RNA complex through the transition state. The differences in K_m values for minihelix^{Ala} and G3·U70,A73 minihelix^{Cys} are within experimental error.

We investigated an A73 minihelix^{Cys} variant to determine whether the presence of A73 alone could confer any alanine acceptance. No aminoacylation was observed, even after prolonged incubation with substrate levels of enzyme (Figure 3). Thus, A73 alone cannot confer alanine acceptance on the minihelix^{Cys}, even though it markedly improves the efficiency of aminoacylation of the same minihelix when it encodes G3·U70.

Change of A73 in Minihelix^{Ala} Reduces the Extent and Rate of Aminoacylation. The marked effect on amino-

Table I: Kinetic Parameters for Aminoacylation with Alanine of Some Minihelix RNAs and tRNAs at pH 7.5, 37 °C[a]

RNA	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
$tRNA^{Ala}$ (*E. coli*)[b]	0.93	2.1	4.4×10^5
G3·C70 $tRNA^{Ala}$			(0)[c,d]
A3·U70 $tRNA^{Ala}$			(0)[c,d]
U3·G70 $tRNA^{Ala}$			(0)[c,d]
G3·U70 $tRNA^{Tyr}$	0.60	14.0	4.3×10^4[e]
$minihelix^{Ala}$ (with U8)	0.91	9.1	1.0×10^5
$minihelix^{Ala}$ (without U8)	0.90	7.5	1.2×10^5
G3·C70 $minihelix^{Ala}$			(0)[c]
U3,U70 $minihelix^{Ala}$			(0)[c]
G3,G70 $minihelix^{Ala}$			(0)[c]
$minihelix^{Cys}$			(0)[c]
G3·U70,A73 $minihelix^{Cys}$	0.28	8.8	3.2×10^4
G3·U70 $minihelix^{Tyr}$	0.48	8.8	5.3×10^4[f]

[a] The minihelices are shown in Figure 1. [b] Data for $tRNA^{Ala}$ and $minihelix^{Ala}$ (with U8) were independently determined in this study and are comparable with those reported by Francklyn and Schimmel (1989). [c] No aminoacylation detected after 30 min of incubation with substrate levels of enzyme. [d] From Park et al. (1989). [e] From Hou and Schimmel (1989b). [f] Data from Francklyn and Schimmel (1989).

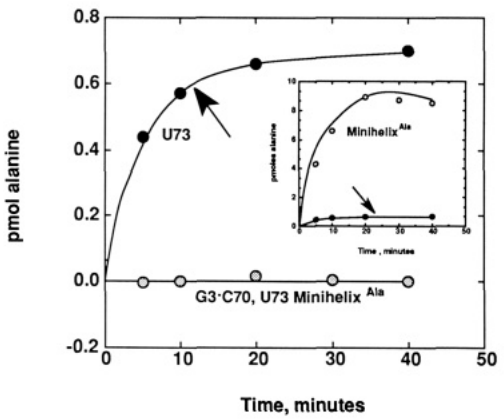

FIGURE 4: Aminoacylation with alanine of $minihelix^{Ala}$ and of position 73 variants. The assays were carried out as described under Materials and Methods. The RNA concentrations for these experiments were as follows: U73, 2 μM; G3·C70,U73, 1 μM; $minihelix^{Ala}$, 0.5 μM. Alanine tRNA synthetase was added to a final concentration of 0.75 μM for all substrates except wild-type minihelix, to which enzyme was added to a final concentration of 75 nM. The inset compares the aminoacylation of $minihelix^{Ala}$ with the U73 variant. The incorporation of alanine per 18-μL reaction aliquot is given on the ordinate axis.

acylation with alanine brought about by changing U73 to A in $minihelix^{Cys}$ prompted us to inquire whether the effect of A73 is specific for $minihelix^{Cys}$ or if the effect of A73 is more general. As part of the original studies that identified G3·U70 as an alanine determinant, U, C, and G substitutions of A73 in $tRNA^{Ala}$ were shown to be tolerated in vivo. In a more detailed study of the U73 suppressor, only alanine was detected as the amino acid inserted at the suppressed amber codon of a dihydrofolate reductase mRNA. Moreover, the efficiency of suppression of a *trpA* (*UAG15*) amber allele was within a factor of 2 of that of the wild-type A73 species (Hou & Schimmel, 1988). By these criteria, therefore, U73 $tRNA^{Ala}$ is an effective alanine-specific tRNA in vivo.

An alanine minihelix that has an A73→U substitution is still charged with alanine, albeit at a much reduced efficiency (Figure 4). Substitution of the other nucleotides (C, G) at position 73 also reduces the extent and rate of aminoacylation (Figure 5) of those substrates that encode G3·U70. On the other hand, G3·C70 $minihelix^{Ala}$ is not aminoacylated, even though it retains A73 (Francklyn & Schimmel, 1989). The implication is that A73 alone cannot specify alanine acceptance

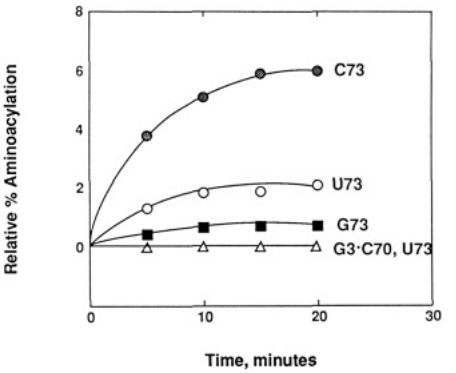

FIGURE 5: Aminoacylation of $minihelix^{Ala}$ derivatives that differ at position 73. The assays were carried out as described under Materials and Methods. The RNA concentrations in these experiments were 10 μM, and the concentration of alanine tRNA synthetase was 0.35 μM. The aminoacylation of each of these substrates is expressed as the percentage of the quantitatively aminoacylated wild-type $minihelix^{Ala}$.

and that the aminoacylation of variants that have nucleotides other than A at position 73 is due to the presence of the G3·U70 base pair.

The results suggest that the effect of A73 in the context of $minihelix^{Ala}$ is similar to its effect in the context of G3·U70,A73 $minihelix^{Cys}$. It enhances aminoacylation of those substrates that encode G3·U70, and this effect is not limited to a particular sequence context.

Discussion

The U73 $tRNA^{Ala}$ and G3·U70 $tRNA^{Cys}$ variants are efficient alanine-inserting suppressors that are not detectably mischarged in vivo (Hou & Schimmel, 1988). While each minihelix version of these tRNAs can be aminoacylated with alanine in vitro, the efficiency is low (Figures 3 and 4). The lack of correlation between efficiency of amber suppression in vivo and aminoacylation efficiency in vitro is striking. The reason for this discrepancy is not known. However, amber suppression in vivo is a more complex phenomenon that is dependent on many more interactions than the isolated synthetase–tRNA interaction in vitro. One difference between in vivo and in vitro systems is that of competition between synthetases for the same tRNA substrate (Swanson et al., 1988; Hou & Schimmel, 1989b). For example, G3·U70 $tRNA^{Tyr}$ can be aminoacylated with either alanine or tyrosine in vitro, depending on which synthetase is added to the reaction mixture. However, the nature of the amino acid that is attached in vivo depends on the relative levels of alanine versus tyrosine tRNA synthetase and on the respective kinetic parameters for the cognate and variant substrates. A complete switch of identity between tyrosine and alanine for G3·U70 $tRNA^{Tyr}$ can be achieved in vivo by manipulation of the relative levels of the two enzymes (Hou & Schimmel, 1989b). However, while the effects of competition are now partly understood, they do not seem to explain the difference between the in vivo and in vitro efficiencies of U73 $tRNA^{Ala}$ and G3·U70 $tRNA^{Cys}$.

More likely, the efficiency of aminoacylation for most substrates is not the limiting factor for amber suppression in vivo. Thus, large perturbations in aminoacylation efficiency can be tolerated without loss of efficient amber suppression. Perhaps a weakly acylated substrate can be sequestered and even accumulated in vivo, because the aminoacylated product is rapidly removed from the charging reaction through binding to the elongation factor EF-Tu. These considerations would indicate that, in the screening of tRNA sequence variants for their suppression in vivo, particular attention should be paid

to those variants that are poor suppressors. Provided that they are not defective in synthesis or stability, these suppressors are likely to be exceptionally poor substrates for the relevant aminoacyl-tRNA synthetase. For example, the position 3·70 variants (such as the U3,U70) studied by McClain et al. (1988) are too weak to suppress the alanine-requiring *trpA* (*UAG234*) amber allele. [This same allele is efficiently suppressed by the U73 $tRNA^{Ala}$ and G3·U70 $tRNA^{Cys}$ variants (Hou & Schimmel, 1988).] The defective suppression by these variants suggests that they are severely reduced in aminoacylation efficiency in vivo, and the failure to observe aminoacylation in vitro of the U3,U70 and G3,G70 minihelix variants, in addition to other sequence combinations at position 3·70, is consistent with this interpretation.

The data reported here show that the contribution to alanine identity of the base pair at 3·70 is cis dominant to the nucleotide at position 73. Nevertheless, even though it cannot compensate for the deleterious effect of other sequences at the 3·70 position, A73 is an effective enhancer of the aminoacylation of those substrates that encode G3·U70. The mechanism by which this base enhances the efficiency of charging by alanine tRNA synthetase is not known. In the X-ray structure of the complex formed between glutamine tRNA synthetase and $tRNA^{Gln}$, the exocyclic 2-amino group of the guanine ring of G73 (the "wild-type" position 73 nucleotide for $tRNA^{Gln}$) is hydrogen bonded to the internucleotide phosphate between C71 and A72. This stabilizes a conformational change in the 3′ end of the tRNA, and only a G at this location is able to make this specific interaction (Rould et al., 1989). Thus, the position 73 nucleotide exerts its influence indirectly through an interaction within the tRNA. The relevance of this observation to other tRNAs, such as $tRNA^{Ala}$, remains to be determined. However, the requirement for a specific nucleotide at position 73 is not a general phenomenon because, among other examples, naturally occurring *E. coli* $tRNA^{Arg}$ (Sprinzl et al., 1989) isoacceptors have A73 or G73 but are charged by the same enzyme and because artificial permutations of N73 in *E. coli* initiator $tRNA^{Met}$ do not have a major effect on aminoacylation (Uemura et al., 1982).

The results presented here do not resolve the question of the molecular basis of recognition of the G3·U70 base pair. Unlike B-form DNA, where recognition of specific bases occurs by interaction of protein side chains with functional groups in the major groove (Jordan & Pabo, 1988), access to the base pairs in an A-form RNA helix is through the minor groove [cf. Rould et al. (1989)]. If the G3·U70 base pair is in the wobble configuration, as observed for a G4·U69 base pair in the acceptor helix of yeast $tRNA^{Phe}$ (Kim et al., 1974; Robertus et al., 1974), then the exocyclic 2-amino group of guanosine is not hydrogen bonded and projects into the minor groove. Thus, this amino group is available for interaction with a complementary group on the enzyme. In the crystal structure of *E. coli* $tRNA^{Gln}$ with Gln-tRNA synthetase, a side-chain carboxyl from Asp235 hydrogen bonds to the 2-amino group of G3, which makes a normal Watson–Crick base pair with C70 (Rould et al., 1989). We imagined that the G3,G70 variant of $minihelix^{Ala}$ might be at least weakly aminoacylated, because the 2-amino group would be available in that molecule. No aminoacylation was detected, even after a 30-min incubation with substrate levels of enzyme (Figure 2). Likewise, the G3·C70 variant was not aminoacylated (Francklyn & Schimmel, 1989). Thus, if the 2-amino group of G3 is important for recognition by alanine tRNA synthetase, then there is a strict requirement for presentation in the context of a G3,U70 base pair. Likewise, because substrates that encode A3·U70 and U3,U70 cannot be acylated, any recognition of the 4-keto group of U70 in the major groove has a requirement for presentation as G3·U70.

The relatively small differences between the kinetic parameters for the aminoacylation of G3·U70,A73 $minihelix^{Cys}$, G3·U70 $minihelix^{Tyr}$, and $minihelix^{Ala}$ might be due to small variations in the helix parameters associated with the different sequences that are proximal to G3·U70. A greater variation on helix parameters could be produced by constitution of a minihelix or tDNA (Kahn & Roe, 1988) with the parameters of B-form DNA, where access to the minor groove is limited. In addition to the structural analysis of a complex, the behavior of this and other variants will provide further insight into the parameters that determine recognition of G3·U70 by alanine tRNA synthetase.

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Serum-Induced Leakage of Negatively Charged Liposomes at Nanomolar Lipid Concentrations†

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ABSTRACT: An enzyme inhibition assay was developed to determine methotrexate-γ-aspartate leakage from liposomes at lipid concentrations as low as 43 nM phospholipid. When negatively charged liposomes prepared with phosphatidylglycerol/cholesterol 67:33 or phosphatidylinositol/cholesterol 67:33 were incubated in 10% (v/v) newborn calf serum, they leaked over 90% of their contents in 2 min. In contrast, liposomes prepared from phosphatidylcholine/cholesterol 67:33 leaked 18% of their contents under the same conditions. The amount of negative charge required to induce liposome leakage was determined by preparing liposomes with varying amounts of phosphatidylglycerol and phosphatidylcholine. Extensive leakage was observed only from liposomes prepared with greater than 50 mol of phosphatidylglycerol per 100 mol of phospholipid. The effect of the phase transition temperature on leakage of negatively charged liposomes in 10% (v/v) serum was investigated by using a series of phosphatidylglycerols with varying acyl chain lengths. Liposomes prepared from distearoylphosphatidylglycerol or dipalmitoylphosphatidylglycerol leaked less than 18% of their contents in 10% serum, whereas liposomes prepared with dilauroylphosphatidylglycerol or unsaturated lipids leaked more than 70% of their contents. Lipoprotein removal from serum followed by treatment with lipid to remove residual apoproteins reduced the leakage from phosphatidylglycerol liposomes in 10% serum. Phosphatidylglycerol liposomes leaked 73% in the presence of human low-density lipoproteins, but only 29% in the presence of bovine apolipoprotein A-I, and 25% in the presence of human high-density lipoproteins. Phosphatidylglycerol/cholesterol and phosphatidylserine/cholesterol liposomes leaked 67% in 4 mg/mL bovine serum albumin purified by cold ethanol extraction. The leakage of liposomes in albumin solutions could be substantially reduced by treating the albumin with lipid. The pronounced effect of ionic strength on leakage of negatively charged liposomes in albumin solutions indicated that apolipoprotein-induced leakage of liposomes is mediated in part by electrostatic interactions.

Liposomes have been proposed as carriers for the delivery of molecules to cells (Gregoriadis & Ryman, 1972). They have proved particularly effective for promoting intracellular delivery of molecules that do not penetrate cells readily (Heath et al., 1985a). Studies of the delivery of such molecules have demonstrated that adsorptive endocytosis is the predominant mechanism of delivery, as evidenced by the inhibition of delivery by lysosomotropic agents (Heath et al., 1985b). This mechanism of delivery requires that liposomes retain their contents until endocytosis occurs. Consequently, the leakage of liposomes in serum would be expected to reduce drug delivery, and should be minimized in order to optimize liposome-mediated drug delivery.

In order to gain useful information from leakage studies, it is important to design experiments carefully so that the conditions employed for liposome leakage experiments match the conditions used during drug delivery experiments. Previously we have shown that fluoroorotic acid is a more effective inhibitor of cell division and contraction when it is encapsulated in dipalmitoylphosphatidylglycerol (DPPG)[1] liposomes than it is when encapsulated in EPG liposomes, presumably because DPPG liposomes are more stable than EPG liposomes (Heath et al., 1987). However, leakage experiments using dialysis techniques indicated that DPPG liposomes were not more stable than EPG liposomes.

The most prominent difference in the experimental conditions between liposomes leakage determinations and liposome drug delivery is the ratio of serum protein to liposomal lipids. Liposome drug delivery in vitro often uses lipid concentrations of 60–100 nM in 10% serum (Heath et al., 1985b). Lipid concentrations in leakage experiments are often micromolar to millimolar in serum or plasma (Allen & Cleland, 1980; Guo et al., 1980; Kirby et al., 1980; Heath et al., 1987; Mayhew et al., 1979; Senior et al., 1985). Therefore, to determine the effects of lipid charge and phase transition temperature on

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[1] Abbreviations: DLPG, dilauroylphosphatidylglycerol; DMPC, dimyristoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPtPG, dipentadecanoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PG, phosphatidylglycerol; PI, soybean phosphatidylinositol; PS, bovine brain phosphatidylserine; SUV, small unilamellar vesicle(s); HDL, high-density lipoprotein(s); LDL, low-density lipoprotein(s); apo A-I, apolipoprotein A-I.

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